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Temperature Dependence of Partial Volumes of Proteins

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Synopsis

The change of the apparent partial specific volumes of egg albumin, bovine serum albumin, bovine methemoglobin, β-lactoglobulin, and lysozyme with temperature through the thermal transitions of the proteins have been measured with dilatometers. Four regions in the plot of the apparent partial specific volumes against temperature can be recognized: (*1*) linear sections extending from 25°C up to 45–50°C; (*2*) a decrease in slope between 50°C and 60°C; (*3*) a sharp increase in slope with increasing temperature coinciding with the appearance of heat coagulation of the protein and followed by (*4*) a decrease in the slope. The return of the protein samples to 25°C yields linear relations between the apparent partial specific volumes of the heat-denatured proteins and the decreasing temperature.

INTRODUCTION

The partial specific volumes of proteins as a function of temperature have been reported.[1,2] However, these investigations have not been as extensive as could be wished. More especially, the volume change suffered by a protein upon heat denaturation has been insufficiently explored although Holcomb and Van Holde[3] have studied the dependence of the partial specific volume of ribonuclease on temperature through the thermal transition of this protein.

Dilatometers have been used in the present research to measure the volume change of protein solutions with increasing temperature; the measurements extending through the thermal transitions of the proteins. These data permit the calculation of the change of the apparent partial specific volumes of the proteins as a function of the temperature.

EXPERIMENTAL

Three dilatometers of pyrex glass with similar dimensions have been used; essentially they were pycnometers with extended stems. For example, one of the dilatometers, had a volume of 4.6221 ml with a stem of 30 cm whose inner diameter was 0.1117 cm. One cm of height in the capillary corresponds to 0.00794 ml.

The dilatometer was immersed in a vigorously stirred water bath with about 5 cm of the stem extending above the surface of the water. The

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dilatometer was clamped in a vertical position and the level of the meniscus in the capillary observed with a cathetometer. The temperature was raised stepwise and about 15 min. was allowed for the dilatometer to equilibrate after the bath had attained the desired temperature. A Yellow Springs thermistor whose mechanical relay had been replaced by a solid-state relay was used to control the temperature. The thermistor was calibrated with a series of Brooklyn Thermometer Co. standard thermometers. The dilatometers were calibrated with water using Kell's density for water.[4]

The experimental procedure for a measurement was as follows: The empty disassembled dilatometer was weighed. Protein solution which had been previously deaerated under vacuum was added to the bulb of the dilatometer which was stoppered and weighed thus giving the weight of the protein solution. The bulb of the dilatometer was filled with cetane and the stem of the dilatometer inserted and reweighed thus giving the weight of the cetane. The dilatometer was placed in the water bath at 25°C and the level of the cetane in the capillary observed with the cathetometer as the temperature was raised. All measurements were made in the reference to the 25°C reading.

The apparent partial specific volume of the protein is given by the expression:

$$\bar{V}_a = \frac{1 - W_1 V_1}{W_2} \tag{1}$$

where W_1 and W_2 are the weights of solvent and proteins respectively, in one ml of solution and V_1 is the specific volume of water.

If the apparent partial specific volume at any temperature is compared with that at 25° Eq. (1) can be converted by simple algebra to:

$$\Delta \bar{V}_a = \frac{\Delta V_s}{W_p} - \frac{W_{H_2O} \Delta V_1}{W_p} \tag{2}$$

where $\Delta \bar{V}_a$ is the change of the partial specific volume of the protein relative to the volume at 25°C. ΔV_s is the corresponding change of the volume of the protein solution and ΔV_1 is the change of the specific volume of water. W_p and W_{H_2O} are the weights of the protein and of the water, respectively, in the protein solution in the dilatometer.

Egg albumin was three times crystallized from fresh hens' eggs with sodium sulfate and dialyzed against water. A portion of this solution was lyophilized and placed over 95% sulfuric acid in a vacuum desiccator for about 50 hr. An aliquot of the sample was dried overnight at 105°C in a vacuum oven. It contained 0.0028 g H_2O per g of egg albumin. This material is referred to as dry egg albumin. The β-lactoglobulin was prepared from raw cows' milk by a method previously described.[5] The crystals were dissolved in 0.1*M* NaCl and dialyzed against 0.1*M* NaCl. The change of the specific volume of the dialyzate with temperature was used in the calculation of the change of the apparent partial specific volume of the

protein. The bovine hemoglobin, bovine serum albumin, and egg white lysozyme were all crystalline products from Sigma Chemical Company.

The hemoglobin was treated with double the equivalent quantity of potassium ferricyanide to produce methemoglobin. The protein was dialyzed against 0.1*M* NaCl and then against water to remove the remaining ferri- and ferro-cyanide. The bovine serum albumin was dialyzed against water before use. The lysozyme was recrystallized twice[6] and the suspension of crystals adjusted to pH 8.5 and dialyzed against water; the crystals dissolved. In all cases the concentrations of the protein solutions have been determined by dry weight at 105°C in a vacuum oven over night.

Cetane was a purified product from Humphry-Wilkinson Inc.

RESULTS

The experimental data are extensive and have been reduced to manageable proportions by calculating the change of the apparent partial specific volumes of the proteins by the use of Eq. (2). The results of these calculations for increasing temperatures are shown in Table I and for decreasing temperatures in Table II. The proteins are identified by column numbers.

TABLE I
Increase of Apparent Partial Specific Volumes of Proteins with Temperature

Temp. (°C)	Column numbers[a]						
	1	*2*	*3*	*4*	*5*	*6*	*7*
25.06	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
30.03	0.0018	0.0019	0.00042	0.0018	0.0017	0.0014	0.0018
35.05	0.0038	0.0037	0.00078	0.0037	0.0034	0.0033	0.0037
40.09	0.0055	0.0055	0.00114	0.0057	0.0049	0.0052	0.0055
45.12	0.0075	0.0071	0.00193	0.0075	0.0061	0.0065	0.0074
50.13	0.0091	0.0086	0.00241	0.0090	0.0077	0.0078	0.0089
55.13	0.0106	0.0101	0.00278	0.0107	0.0088	0.0090	0.0106
56.13	0.0107	0.0105	0.00281	0.0109	0.0090	0.0082	0.0106
58.13	0.0112	0.0109	0.00268	0.0113	0.0091	0.0094	0.0112
60.23	0.0124	0.0120	0.00296	0.0120	0.0097	0.0103	0.0119
62.20	0.0138	0.0130	0.00306	0.0128	0.0105	0.0105	0.0125
64.17	0.0156	0.0147	0.00314	0.0135	0.0112	0.0112	0.0131
66.13	0.0173	0.0167	0.00332	0.0136	0.0120	0.0119	0.0137
68.08	0.0197	0.0193	0.00361	0.0149	0.0139	0.0127	0.0146
70.02	0.0210	0.0206	0.00397	0.0154	0.0152	0.0131	0.0152
71.96	0.0224	0.0220	0.00422	0.0162	0.0175	0.0142	0.0162
73.90	0.0232	0.0229	0.00437	0.0168	0.0180	0.0152	0.0181
75.83	0.0243	0.0242	—	0.0179	0.0188	0.0160	0.0227
77.78	0.0250	0.0249	—	0.0187	0.0198	0.0172	0.0251
79.71	0.0258	0.0256	—	0.0194	0.0197	0.0175	0.0259
81.66	0.0269	0.0265	—	0.0206	0.0215	0.0183	0.0269
83.60	0.0276	0.0272	—	0.0209	0.0224	0.0196	0.0280
84.57	—	0.0277	—	0.0213	—	—	0.0283

[a] Columns *1*, *2*, and *3*, egg albumin; *4*, bovine serum albumin; *5*, lysozyme; *6*, methemoglobin; *7*, β-lactoglobulin.

TABLE II
Decrease of the Apparent Partial Specific Volumes of Proteins with Decreasing Temperature

Temp. (°C)	Column numbers[a]						
	1	*2*	*3*	*4*	*5*	*6*	*7*
83.60	0.0276	0.0272	—	0.0209	0.0224	0.0196	0.0280
79.71	—	0.0259	—	0.0199	—		
74.88	—	0.0239	—	0.0173	0.0191	0.0183	0.0239
70.02	—	0.0221	0.0039	0.0155	—	—	—
65.15	0.0202	0.0200	0.0032	0.0136	0.0166	0.0149	0.0207
60.23	—	0.0180	0.0027	0.0116	—	—	—
55.13	—	0.0156	0.0023	0.0092	0.0142	0.0108	0.0169
50.13	—	0.0140	0.0018	0.0073	—	—	—
45.12	0.0119	0.0118	0.0014	0.0051	0.0110	0.0075	0.0130
40.09	—	0.0098	0.0007	0.0029	—	—	—
35.05	—	0.0080	−0.0002	0.0009	0.0073	0.0037	0.0088
30.03	—	0.0059	−0.0004	−0.0012	—	—	—

[a] Columns *1*, *2*, and *3*, egg albumin; *4*, bovine serum albumin; *5*, lysozyme; *6*, methemoglobin; *7*, β-lactoglobulin.

The concentrations of the proteins, expressed in grams of protein per gram of solution, corresponding to the numbered columns are as follows: (*1*) 0.1510, egg albumin; (*2*) 0.2509, egg albumin; (*3*) dry egg albumin; (*4*) 0.1521, bovine serum albumin; (*5*) 0.1320, lysozyme; (*6*) 0.08015, methemoglobin; (*7*) 0.1682, β-lactoglobulin.

DISCUSSION

The accuracy of the calculated values for $\Delta \bar{V}_a$ of the protein depends on the accuracy of eight measured quantities which are: (1) the weight of cetane; (2) the weight of the protein solution is the dilatometer; (3) the dry weight concentration of the protein solutions; (4) the change in the specific volume of water with temperature; (5) the measured temperature; (6) the volume–height calibration of the capillary with water; (7) the expansion of cetane with temperature; (8) the level of cetane in the capillary.

The errors involved in items 1, 2, 3, and 4 above are certainly minor compared with those inherent in items 5, 6, 7, and 8. An error in the temperature results in the selection of an incorrect value for the specific volume of water. For example, 0.1510 g egg albumin per gram of solution at 55.15°C yields a calculated value of $\Delta \bar{V}_a$ (see Table I) of 0.01061. Had we made an error of ±0.05°C in our reading of the temperature, this would have led to an error in the specific volume of water which would have produced ±1.32% error in the calculated apparent partial specific volume of the protein. Our temperature error is more likely about ±0.02°C. As stated in the section titled Experimental, in one of our dilatometers 1 cm in height of the capillary corresponds to 0.009794 ml. The standard devia-

tion of twenty-four readings in the calibration was ±0.000089 and the standard deviation of the mean of the series was 0.000018. These standard deviations for the 0.1510 g egg albumin per gram of solution at 55.15°C produces errors of 6.6% and of 1.3% in the calculated individual $\Delta\bar{V}_a$ values for the protein and in the systematic error, respectively. The expansion of the cetane with temperature was measured in the dilatometer filled with cetane using the water calibration of the volume–height relation of the capillary. The errors involved in the cetane expansion coefficient are likely of the same magnitude as for the original water calibration. However, since the weight of cetane used with the protein solution was about 0.30 that of the dilatometer filled with cetane, the error in the $\bar{V}_a$ value for the protein would be correspondingly reduced. The maximum possible error we could make in cathetometer measurement of the level of cetane in the capillary would be ±0.005 cm. For the 0.1510 g egg albumin per gram of solution at 55.15°C, this error in the reading of the level of the meniscus would correspond to an error of ±0.85% in $\Delta\bar{V}_a$ for the protein. Comparing the calculated $\bar{V}_a$ values for 0.1510 g egg albumin per gram of solution obtained by one operator (K.B.) with those obtained with 0.2509 g egg albumin per gram of solution of another operator (H.B.B.) over the entire temperature range (twenty-one values; see Table I) gives an average percent deviation of 2.75. Likely, there is a certain amount of cancellation of errors.

Bovine serum albumin from 25°C up to about 45°C yields a linear relation between $\Delta\bar{V}_a$ and temperature. The slope of this line is 3.75×10^{-4} ml/g/degree. This value agrees much more closely with that of Hunter[1] for this protein (3.65×10^{-4} ml/g/degree) than it does with our previous value[2] reported for this protein using a specific gravity balance (4.70×10^{-4} ml/g/degree).

The values for $\bar{V}_a$ shown in Table I have been plotted on large-scale graphs against temperature and the slopes of the lines estimated. There is admittedly a subjective element in the determination of these slopes. However, anyone wishing to make his own estimates can use the data in Table I. Our estimates of the slopes are shown in Figures 1 and 2.

Characteristically, $\Delta\bar{V}_a$ is linear in respect to temperature from 25°C up to 45–50°C. The slopes of these lines for the various proteins are 3.03×10^{-4}, 3.75×10^{-4}, 3.50×10^{-4}, 3.75×10^{-4}, and 3.05×10^{-4} all expressed as ml/g/degree for lysozyme, bovine serum albumin, egg albumin, β-lactoglobulin, and methemoglobin, respectively. There is a departure from linearity starting between 45 and 50°C with decreasing slopes; the slopes go through minima at about 55°C. Above this temperature, the slopes increase rapidly with increasing temperature. Lysozyme, β-lactoglobulin, and bovine serum albumin exhibit plateaus in their slopes starting at about 60°C and extending up to 66–69°C. With the exception of bovine serum albumin, the slopes go through maxima with increasing temperature. Both egg albumin and lysozyme show pronounced maxima; that for β-lactoglobulin is especially conspicuous.

The results reported by Holcomb and Van Holde[3] on the apparent partial specific volume of ribonuclease as a function of temperature resemble our observations. Their measurements were made in 0.01M potassium phthalate and 0.15M KCl to which HCl was added to yield a pH of 2.80.

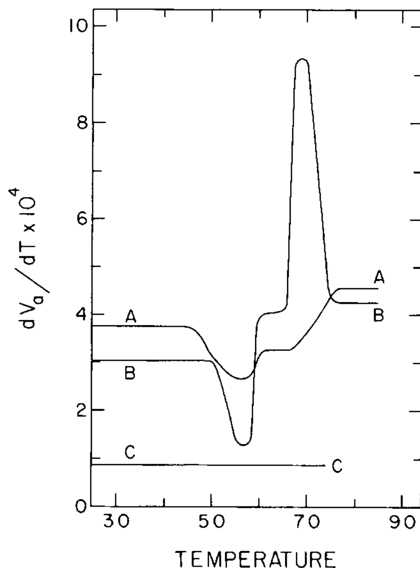

Fig. 1. Estimated slopes of the change of the apparent partial specific volumes of proteins with temperature plotted against the temperature (°C). A, bovine serum albumin, B, lysozyme; C, dry egg albumin.

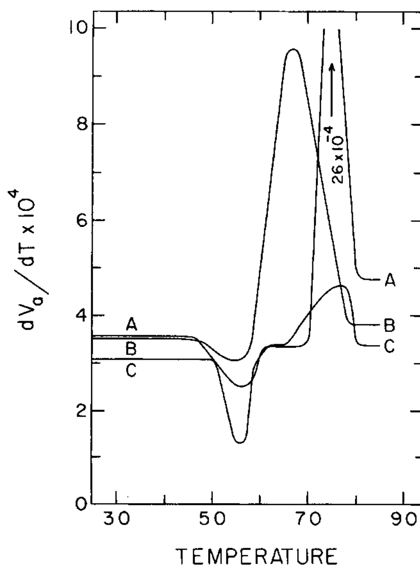

Fig. 2. Estimated slopes of the change of the apparent partial specific volumes of proteins with temperature plotted against the temperature (°C). A, β-lactoglobulin; B, egg albumin; C, methemoglobin.

At this pH, the midpoint of the thermal transition of ribonuclease was 45°C. They found a linear dependence of the partial specific volume from about 15°C to about 32°C with a slope of 4.5×10^{-4} ml/g/degree. The slope of the plot of the partial specific volume against increasing temperature diminished starting at about 32°C extending up to about 55°C. At this temperature the slope greatly increased without exhibiting a maximum. The behavior of ribonuclease at pH 2.80, therefore, resembles more nearly that observed by us for bovine serum albumin.

Evidently, the course of the volume changes of proteins with temperature is complex. We suggest the following tentative explanation for the series of events associated with the increase in the partial volumes of proteins with increasing temperature. Over the linear part of the plots of $\Delta \bar{V}_a$ against temperature starting at 25°C, the increased slopes over that of the anhydrous protein (egg albumin) are likely due mainly to the release of adsorbed water from the proteins. The hydration of proteins is exothermic[7] and the density of adsorbed water is significantly greater than that of water in bulk.[8] In addition to the release of water, the protein also expands with increasing temperature. At about 55°C there are minima in the $d\bar{V}_a/dT$ against temperature for all of the proteins (see Figs. 1 and 2). If we make the simple assumption that the minima are due to the disappearance of water molecules into the interior of the protein molecules, it is possible to estimate the number of water molecules involved. We make these estimates by comparing the observed $\Delta \bar{V}_a$ values with those obtained from the extension of the linear portions of the $\Delta \bar{V}_a$ against temperature plots. These estimates expressed in moles of water per mole of protein are as follows: 0.8, 4.9, 2.1, 3.6, and 1.1 for egg albumin, bovine serum albumin, β-lactoglobulin, methemoglobin, and lysozyme, respectively. Evidently, only a few water molecules per mole of protein are involved.

Privalov, Khechinashvili, and Atanasov[9] studied the thermal transitions of chymotrypsinogen, ribonuclease, and myoglobin using a differential thermal analyzer. They state that they observed a pre-denaturational stage in which the protein partial heat capacity changes are probably connected to a labilization of the native protein structure and secondly a denaturational stage representing a single-step transition into a state with a higher enthalpy. It is not unlikely that the minima in our $d\bar{V}_a/dT$ against temperature curves are related to the pre-denaturational stage of the Russian workers.

We have observed in cleaning our dilatometers of protein after having raised the temperature to 84°C and returning to room temperature that β-lactoglobulin formed an exceedingly stiff coagulum. The coagula of egg albumin and of lysozyme were somewhat less stiff whereas those of methemoglobin and especially that of bovine serum albumin were practically liquid. It appears likely that the large maxima in the $d\bar{V}_a/dT$ against temperature plots for β-lactoglobulin, egg albumin, and for lysozyme are due to the considerable interaction and cross bonding between the heat-denatured forms of these molecules. The cross-bonding between the pro-

tein molecules leads to release of adsorbed water from the protein giving rise to the maxima.

In all cases, as shown in Table II the return of the protein samples to 25°C yields linear relations between the apparent partial specific volumes of the heat denatured proteins and decreasing temperature. Plots of $\Delta \bar{V}_a$ against temperature gave the following slopes expressed as ml/g/degree: 4.04×10^{-4}, 4.12×10^{-4}, 3.00×10^{-4}, 3.67×10^{-4}, and 3.80×10^{-4} for egg albumin, bovine serum albumin, lysozyme, methemoglobin, and β-lactoglobulin, respectively. Actually, a fair amount of experimental work has been done which is not being submitted for publication. We determined the apparent partial specific volumes of both egg albumin and of bovine serum albumin as a function of temperature using ordinary pycnometers. The experimental variations especially at higher temperatures were, however, too large, but the results were entirely consistent with those later obtained with dilatometers.

The dilatometric experiments which are not being reported are: *(1)* 0.1455 g egg albumin per gram of solution at pH 3.34 (pH adjusted with HCl). The thermal transition, due to the lower pH, occurred at a lower temperature otherwise the results were the same as those for the isoelectric sample. *(2)* 0.1468 g egg albumin per gram of solution at pH 6.42 (pH adjusted with NaOH). This result practically duplicated those obtained with the isoelectric sample. *(3)* 0.05471 g egg albumin per gram of solution (isoelectric). As was to be expected, this solution showed greater experimental variation than did the more concentrated solutions. Otherwise, the results were the same as for the more concentrated solutions.

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